# Diversity and Patterns of Regulation of Nicotinic Receptor Subtypes[a]

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## EVIDENCE FOR nAChR SUBTYPE AND SUBUNIT DIVERSITY

Nicotinic acetylcholine receptors (nAChR) are now known to exist as a heterogenous group of macromolecules.[1–4] In retrospect, it is surprising that the concept of nAChR diversity was not more rapidly and widely accepted. Comparative pharmacological studies dating from the times of Dale, Paton, and their contemporaries clearly indicated differences in nicotinic drug actions at the vertebrate neuromuscular junction and at autonomic ganglia.[5–7] Other pharmacological studies also suggested that nAChR subtypes distinct from those found in muscle and ganglia exist in the central nervous system (CNS) as well, based on differing sensitivity to small nicotinic agonists or antagonists.[8–9] More recent studies extended those observations to different classes of neurotoxins, such as the bungarotoxins and neosurugatoxin, and to more reduced preparations.[2,4,10–12] This evidence for nAChR functional diversity in muscle, ganglia, and brain can only be explained—given that possible complications of pharmacokinetics are excluded—if there is structural diversity of nAChR.

Following the success in elucidation of the structure of muscle-type nAChR from the electric organ and the demonstration that muscle-type nAChR are composed of homologous, but distinct, subunits,[13–14] studies employing protein chemical, radioligand binding, and immunochemical techniques also demonstrated the existence of structurally distinct nAChR subtypes.[2–4] About the time that pharmacological features were being assigned to these distinct entities on the basis of functional and radioligand binding studies, the application of recombinant DNA techniques revealed the existence of a family of homologous, but genetically distinct, nAChR subunits that were expressed not in muscle, but in the nervous system.[2–4,15] Currently, at least 15 different nAChR subunit genes, including 5 expressed in muscle and 10 "neuronal" nAChR subunits, have been identified. Those neuronal nAChR subunits that have tandem cysteine residues near the putative nicotinic ligand-binding active site (i.e., in which features of the ligand-binding domain of the $\alpha 1$ subunit are preserved) are defined as $\alpha$ subunits, whereas those neuronal nAChR subunits that lack the tandem cysteine residues but that retain other features of muscle nAChR subunits are defined as non-$\alpha$ or, more popularly, $\beta$ subunits. Tissue- and/or brain region-specific expression of some of these genes restricts possible assignments to

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identified nAChR subtypes, but work continues to elucidate the rules that define which subunits combine to form unique nAChR subtypes. Nevertheless, it is clear that diversity in nAChR subunit genes provides at least a partial basis for nAChR subtype diversity.

## RELATIONSHIPS BETWEEN DIVERSE nAChR SUBUNITS

Alignment and analysis of some of the deduced amino acid sequences of rat nAChR subunits provide interesting perspectives on possible patterns of nAChR subunit evolution that are presently consistent with known properties and distributions of the products of nAChR subunit genes (FIG. 1, TABLE 1). "Structural" subunits of rat muscle nAChR, which include β1, δ, fetal γ, and adult ε subunits,[14] form a branch in the nAChR subunit phylogenetic tree that appears to have diverged early in evolution from a separate branch that contains the "ligand-binding" α subunits. The fetal γ/adult ε subunit pair shares (a perhaps surprisingly low) 52% amino acid sequence identity, and the δ subunit is 42/47% identical to the γ/ε subunit. However, no more than 40% sequence identity exists between these subunits and the β1 subunit or between β1, γ, δ, or ε subunits and any of the other subunits in the ligand-binding branch.

The α7 subunit, which is a constituent of at least some of the neuronal/nicotinic α-bungarotoxin binding sites of the CNS and probably also of the autonomic nervous system (ANS),[10,16–18] is the most distant of any of the members of the nAChR subunit

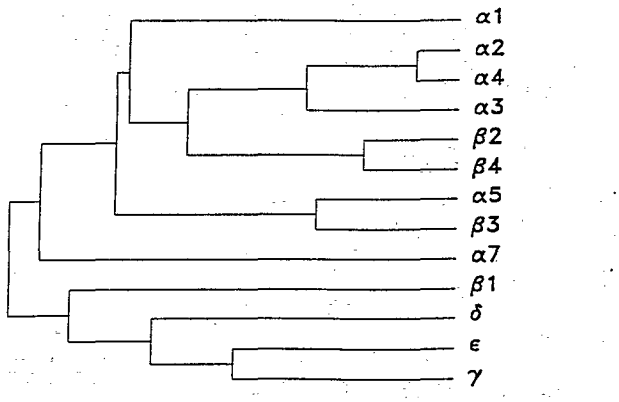


**FIGURE 1.** Dendrogram or phylogenetic tree diagram showing relationships between rat nAChR subunit amino acid sequences. Amino acid sequences for rat nAChR subunits were aligned and analyzed for sequence similarity according to the PCGENE program CLUSTAL using a k-tuple value of 1, a gap penalty of 5, a window size of 10, a filtering level of 2.5, and open gap and unit gap costs of 10. The program fit the indicated sequences to a reference sequence of 677 amino acids. Branch points farthest to the right indicate subunits with highest degrees of identity or identity plus similarity (65 and 73%, respectively, for α2 and α4), whereas branch points to the left on the diagram indicate lower degrees of identity or identity plus similarity (e.g., 32 and 47% for β1, γ, δ, and ε globally versus the other subunits). Although isolation of a rat nAChR α6 subunit gene has been reported, we did not have access to the amino acid sequence, and whereas a chick nAChR α8 subunit gene has been identified, no rat homolog has been described.

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TABLE 1. Percentage Identity/Identity Plus Similarity for Rat nAChR Subunits[a]

| Sub-unit | α1 | α2 | α3 | α4 | α5 | α7 | β1 | β2 | β3 | β4 | γ | δ | ϵ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| α1 | | 46/59 | 51/65 | 51/64 | 42/67 | 38/52 | 35/49 | 43/59 | 42/58 | 40/55 | 32/45 | 36/53 | 31/47 |
| α2 | | | 54/64 | 65/73 | 50/65 | 39/51 | 37/51 | 44/57 | 53/66 | 46/59 | 32/43 | 36/50 | 30/43 |
| α3 | | | | 58/68 | 48/65 | 36/50 | 34/48 | 45/58 | 47/63 | 44/56 | 32/47 | 35/50 | 32/49 |
| α4 | | | | | 52/68 | 36/48 | 37/52 | 50/62 | 53/67 | 48/61 | 33/44 | 34/49 | 30/43 |
| α5 | | | | | | 32/48 | 36/54 | 41/60 | 64/76 | 43/59 | 32/47 | 35/53 | 30/48 |
| α7 | | | | | | | 28/41 | 31/46 | 33/46 | 38/52 | 25/35 | 23/33 | 22/34 |
| β1 | | | | | | | | 40/55 | 36/53 | 38/53 | 40/54 | 35/49 | 35/50 |
| β2 | | | | | | | | | 40/58 | 61/71 | 35/47 | 39/54 | 34/47 |
| β3 | | | | | | | | | | 41/56 | 29/44 | 36/54 | 27/43 |
| β4 | | | | | | | | | | | 37/49 | 39/54 | 33/46 |
| γ | | | | | | | | | | | | 42/53 | 52/65 |
| δ | | | | | | | | | | | | | 47/63 |
| ϵ | | | | | | | | | | | | | |

[a]Table matrix shows (percentage identity)/(percentage identity plus similarity) for indicated rat nAChR subunits. Amino acid sequences for rat nAChR subunits were aligned and analyzed pairwise for identity and similarity according to the PCGENE program ALIGN using the structure-genetic comparison matrix, an open gap cost of 7 and a unit gap cost of 1 where similar amino acid groups are (single letter code) A,S,T; D,E; N,Q; R,K; I,L,M,V; and F,Y,W.

family. It shares less than 40% identity with other members of the ligand-binding branch of nAChR subunits and less than 25% amino acid sequence identity with members of the muscle nAChR structural subunit branch. Perhaps the α7 subunit is just the first member in a third branch or subfamily of rat nAChR subunits that will be shown to also include other, currently unidentified members (the chick α7 subunit shares 72% identity with the chick α8 subunit,[19] but the rat homolog of α8 has not yet been identified).

The α5 (found in both the CNS and ANS) and β3 (found, to date, only in the CNS) pair of subunits share 64% identity and constitute a subunit group of the ligand-binding subunit branch or subfamily.[20–21] It is interesting that no functional role for either of these subunits has yet been found,[2–4] but that they are more closely related to the five nAChR subunits that are known to have functional roles in the ANS and CNS and to the muscle ligand-binding α1 subunit (41–52% and 40–53% identity to α1–4, β2, and β4 for α5 and β3, respectively) than is the α7 subunit.

The muscle nAChR α1 subunit is more like the α2–4 subunits (46–51% identity) than it is like any of the others (31–43% identity to α5, α7, β1–4, γ, δ or ϵ). It seems to have diverged more recently than the α7 subunit (and at about the same time as the α5/β3 and β2/β4 pairs) in evolution from the other truly ligand-binding subunits (α2–4).

The β2/β4 pair of structural subunits, both of which are found in the ANS and CNS, share 61% sequence identity, are 40–50% identical to α1–4 subunits, and are more closely related to the α-bungarotoxin-insensitive, neuronal nAChR α2–4 subunits than to β1, β3, or other muscle nAChR structural subunits. β2 and β4 subunits are distinctive in that they can substitute for the β1 subunit as a functional partner with muscle α1, γ, and δ subunits, and they can combine to form functional nAChR with either α2, α3, or α4 subunits in the *Xenopus* oocyte nAChR expression system,[2–4,15] thereby truly fulfilling part of the definition of structural subunits. However, because they can also influence ligand interactions with neuronal α2–4 subunits, they represent a group of functionally relevant subunits as might be

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expected for structural subunits "displaced" in the ligand-binding branch of the nAChR subunit phylogenetic tree.

Diverging next from the main branch of the ligand-binding subunit subfamily is the α3 subunit, which shares 54–58% identity with α2 and α4 subunits and seems to function as the predominant ligand-binding subunit in autonomic ganglia.[22–25] The α3 subunit is also expressed in a narrowly defined range of brain structures, perhaps as a component of some CNS nAChR that may help to control neurotransmitter release.[2–4,15,26–28] The remaining α2 and α4 subunits share 65% identity and represent a minor and the dominant ligand-binding (high-affinity agonist binding) subunit, respectively, in the rat CNS.

In the phylogenetic tree, α3, α5, and β4 subunits, which are found in a tight gene cluster in the rat or chicken,[20–21] in the human on chromosome 15,[29] and probably in the mouse on chromosome 9,[30] are more closely related to other subunits than to each other, suggesting that translocations to other chromosomes occurred more recently than the tandem duplication of a common ancestor that may have given rise to this cluster of genes. Along with α7 and β2 genes, clustered α3, α5, and β4 subunit genes are expressed in autonomic ganglia and related clonal cells[21–25,31,32] and are coordinately regulated in response to a number of stimuli (see below). Hence, it is perhaps not surprising that they have been found, in chick, to combine in formation of a unique ganglionic nAChR subtype. What is not evident from the data presented in FIGURE 1 and TABLE 1 is the basis for much of the divergence in amino acid sequences between nAChR subunits. All nAChR subunits have similar patterns of amino acid sequence suggesting that they contain an extended, N-terminal, extracellular domain, four transmembrane domains, an extended cytoplasmic loop between the third and fourth transmembrane domains, and an extracellular C-terminal tail. The putative, extended cytoplasmic loops are almost totally unique from one subunit to another not only in amino acid sequence, but also sometimes in length. For example, the rat α4 subunit is over 120 amino acids longer than the α3 subunit in the putative cytoplasmic domain, and the α2/α3 subunit pair shares 54% amino acid identity across the full sequence, 71% identity when the signal sequence and extended cytoplasmic loop are excluded, and only 20% identity through the signal sequence and cytoplasmic loop. Thus, the most unique feature of any nAChR subunit is its cytoplasmic domain.

## FUNCTIONAL RELEVANCE OF nAChR SUBUNIT AND SUBTYPE DIVERSITY

A question that arises from these discoveries is, What is the functional relevance of nAChR subtype and subunit diversity?[2] Part of the answer must derive from the difficulties presented in defining functional roles of nAChR in the ANS and CNS and from recent insights gleaned from those studies.[2–4,33] Advances in understanding neuronal nAChR have come slowly, perhaps because of technical limitations, but also perhaps because some aspects of the functional relevance of nAChR diversity are already evident and run counter to dogma based on our classical understanding of muscle and ganglionic nAChR. For example, some neuronal nAChR desensitize so quickly in the presence of agonists that it now is not surprising that their functions (and perhaps those of other, related nAChR subtypes) escaped detection for so long.[11,12,16,34,35] Some neuronal nAChR may be located only on nerve terminals where their activation may not produce easily detectable currents but where they could have profound effects on neurotransmitter release.[27,28] Some neuronal nAChR may function as ligand-gated $Ca^{2+}$ channels as well as or rather than ligand-gated $Na^{+}$

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channels.[10–12,36,37] Established and more contemporary evidence indicates that sensitivity to drugs, including exogenous agents such as nicotine and endogenous acetylcholine, differs across tissues, cell types, and brain regions (i.e., across nAChR subtypes) and that diverse nAChR subunits play significant roles in the process of drug selectivity.[2,4,15,16,22,23,38–41] From this evidence and these lines of reasoning, it is clear that part of the functional relevance of nAChR diversity is that it allows closely related members of this single class of receptors to have diverse and physiologically relevant functions, ligand sensitivities, and tissue, regional, and cellular/subcellular distributions.

There is at least one example of how nAChR functional diversity can have physiological ramifications and teleological implications. The switch from a fetal to an adult phenotype of muscle nAChR that occurs about the time of motor neuronal innervation of recently differentiated myotubes simply reflects the switch from $\gamma$ to $\epsilon$ subunit expression.[2,42] Nevertheless, fetal, $\gamma$-containing nAChR have lower conductance and longer mean open channel times than do adult, $\epsilon$-containing nAChR, and fetal nAChR are expressed all over the noninnervated myotube surface, whereas adult nAChR expression is restricted to synaptic junctions. We can speculate that this subunit switch became stabilized through evolution because it conferred a functional advantage to developing muscle, in which fetal nAChR initially function as low-gain, poorly time-resolved, innervation sensors distributed widely over the muscle cell, whereas adult nAChR expressed only subsynaptically in innervated muscle function as high-gain elements best designed to respond to rapid chemical transients as would occur at the active neuromotor synapse. Hence, even a modest switch in expression of two closely related subunits contributes to a dramatic remodeling of a critical synapse as well as changes in nAChR function, distribution, and perhaps drug sensitivity.

Another perspective on the functional relevance of nAChR diversity comes from an understanding of the critical roles that nAChR play in nervous system function throughout the organism.[2] They are the exclusive mediators of excitatory neurotransmission at the neuromuscular junction, thereby regulating movement, and at preganglionic-postganglionic synapses, thereby regulating all autonomic activity. Given their widespread localization throughout the brain and just the beginnings of their functional characterization at those sites, neuronal nAChR must share with ionotropic glutamate receptors and serotonin ($5\text{-}HT_3$) receptors the mediation of excitatory neurotransmission that completes brain neuronal circuitry and contributes to higher-order brain processes including perception, cognition, and emotion. Collectively, the functional importance of nAChR centrally and in the periphery suggests that they are ideal targets for the regulation of nervous system function.

Does nAChR diversity allow for diversity in regulation of nAChR expression as well as for diversity in function, ligand selectivity, and distribution?[2] Does diversity in regulation of nAChR expression dovetail with diversity in nAChR and their functions to enhance the potential for plasticity in what we now are beginning to realize is a plastic and dynamic nervous system? If so, then we are only beginning to appreciate the functional relevance of nAChR diversity. We can speculate that the ability of a neuronal cell to express a different nAChR subtype during development or in response to changes in its environment could confer a functional benefit to that cell, just as it seems to be the case for muscle cells expressing fetal versus adult nAChR and subunit genes as a function of innervation state. For example, early in development, a nicotinic cholinoceptive cell may be functioning unconditionally as a circuit element whenever its dendritic nAChR sense release of acetylcholine. If the dendritic tree grows and encounters axons whose transmitters cause dendritic levels of some second messenger to increase, and if the function of the expressed, dendritic

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nAChR is inhibited in the presence of those second messengers, then that cholinoceptive cell may have altered patterns of firing, responding now as a conditional circuit element active only in response to nicotinic stimulation, but not in response to simultaneous nicotinic stimulation and activation of second messenger production. Could such a cell change its pattern of nAChR subunit expression to alleviate second messenger sensitivity? As that cell innervates its targets, or as the cellular dendritic tree and its contacts with retrograde circuit elements are altered, would there be changes in retrograde, anterograde, or extracellular matrix-bound growth factor influences on nAChR expression and, hence, cell function? How would such a change in cell environment be sensed, and how could a message to alter expression of nAChR subunits be transmitted?

## MECHANISMS OF REGULATION OF DIVERSE nAChR SUBTYPES

Here we report a sampling of an ongoing series of studies in this laboratory concerning patterns of nAChR regulation. These studies are designed in part to define some mechanisms of regulation of nAChR expression and function and the roles of nAChR diversity in those processes. We considered the potential roles of diversity (1) in nAChR subunit genes in differential transcriptional control of nAChR expression and (2) in nAChR subunit amino acid sequences in differential posttranslational control of nAChR expression and modification of nAChR function as we described and discussed in detail previously.[2] We also took note of the uniqueness of amino acid sequences in putative cytoplasmic domains across nAChR subunits and for a given subunit across species. Even though sequences for a given subunit across mammalian species typically share 80–90% identity and about 95% identity plus similarity, cross-species differences in sequence most often appear in the cytoplasmic domains and at sites that may have functional relevance. For example, three putative protein kinase C phosphorylation sites are present in the cytoplasmic domain of the rat $\alpha 3$ subunit, but only one of those sites is preserved in the human $\alpha 3$ sequence. The human $\alpha 5$ subunit contains four putative protein kinase C phosphorylation sites in its cytoplasmic domain, but only one of these sites is expressed in the rat $\alpha 5$ subunit sequence. Therefore, we considered the possibility that homologous subunits in different species may be regulated differently. Aside from the possible roles of divergent cytoplasmic domains in conferring differential sensitivity of nAChR subtypes and subunits to actions of interacting receptors altering second messenger activity, protein kinase activity, and nAChR phosphorylation state, we also considered roles that cytoplasmic domains might play in stabilizing nAChR at the cell surface, in targeting nAChR to different locales, and in modulating nAChR function through their interactions with cytoskeletal elements. We also have examined potential roles for nicotinic ligands in the regulation of expression of their own receptors, and of growth factor-mediated effects on nAChR expression.

We have been advocates of the use of clonal cell lines as models for the identification and characterization of diverse nAChR subtypes, and some of our previous (and ongoing) work with clonal cells contributes to continuing development of the concept of nAChR diversity. We now further advocate the use of clonal cell lines as models for studies of mechanisms of nAChR regulation. We argue that clonal cells are well suited for such an application, in that they are homogenous, can be generated in quantities suitable for virtually any type of biochemical application, and can be subjected to electrophysiological or chemical measures of nAChR function. Clonal cells naturally express the same nAChR subtypes as are found in analogous non-neoplastic tissues from which the tumor cells are derived; that such

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expression is under the control of natural and not artificial promoters is particularly relevant to studies of nAChR regulation, which may have a transcriptional component. We found—and will continually be challenged to show—that clonal cells also possess similar, if not identical, intracellular signaling mechanisms as are found in their non-neoplastic analogs. At a minimum, this allows us not only to define mechanisms of nAChR regulation, but also to narrow the realm of possibilities for regulation of a given nAChR subtype by an interacting neurotransmitter, growth factor, or matrix receptor system in a given cell type. Obviously, a future challenge will be to extend our observations to non-neoplastic tissues. However, at present, particularly if the phenomenology of a drug treatment on nAChR in a clonal cell is similar to that for the same drug treatment on a non-neoplastic cellular analog, the intensive work needed to establish mechanisms involved is more expeditiously done using the clonal cell line models.

The three cell lines that we used as models for our studies are (1) the human TE671/RD clone, which expresses α1, β1, γ, and δ subunits in a fetal muscle-type nAChR that mediates α-bungarotoxin (Bgt)-sensitive, nicotinic agonist-gated monovalent cation channel activity and binds [$^3$H]-labeled acetylcholine ([$^3$H]ACh) or [$^{125}$I]-labeled α-bungarotoxin (I-Bgt) with high affinity;[2] (2) the $BC_3H$-1 mouse muscle cell line, which also expresses fetal muscle nAChR subunits as a Bgt-sensitive, fetal, muscle-type nAChR; and (3) and the SH-SY5Y human neuroblastoma, which expresses nAChR α3, α5, α7, β2, and β4 subunits and two types of neuronal nAChR.[22] We use the term neuronal/nicotinic Bgt binding sites (nBgtS) in reference to high-affinity I-Bgt binding sites found in these cells that seem to contain α7 subunits but for which we have found (to date) no evidence for ion channel function, and we use the term ganglia-type nAChR in reference to other neuronal nAChR found in these cells that appear to contain α3 and β4 subunits that mediate both monovalent cation and $Ca^{2+}$ conductances in response to nicotinic agonists in a Bgt-insensitive manner and that bind [$^3$H]ACh with high affinity, also in a Bgt-insensitive manner.[22,43]

## EFFECTS OF CHRONIC NICOTINE TREATMENT

Previous studies from this laboratory have established that chronic nicotine treatment (1 mM) leads to an increase in numbers of muscle-type nAChR (I-Bgt binding sites) in crude membrane preparations of TE671/RD cells that is evident within 6–12 h of drug treatment and reaches and maintains maximal values (2–3-fold increase) after one day and for as long as five days of drug treatment.[44] Other previous work established that nAChR function is lost, however, even during this phase of nicotine-induced up-regulation of nAChR numbers.[45] Phenomenologically, these results are similar to those observed for effects of chronic nicotine treatment on cell ganglia-type nAChR or for chronic nicotine treatment on mouse or rat brain nAChR.[26,44,45] New results shown here indicate that these effects on nAChR numbers do not involve either transient or sustained increases in levels of messenger RNA coding for TE671 cell α1, β1, γ or δ subunits (FIG. 2). We made similar observations using nicotine treatment of primary rat cortical neurons in culture as a model—in this case, in reference to high-affinity [$^3$H]nicotine binding sites and mRNA encoding the α4 and β2 subunits that make up those nAChR, using a lower (1 μM) concentration of chronic nicotine.[46] Other previous work using an intact mouse model showed that 10 days of chronic nicotine infusion, which is adequate to affect an increase in [$^3$H]nicotine binding sites, failed to induce a sustained increase in nAChR α4 or β2 subunit mRNA.[26] Our results add new information that excludes a

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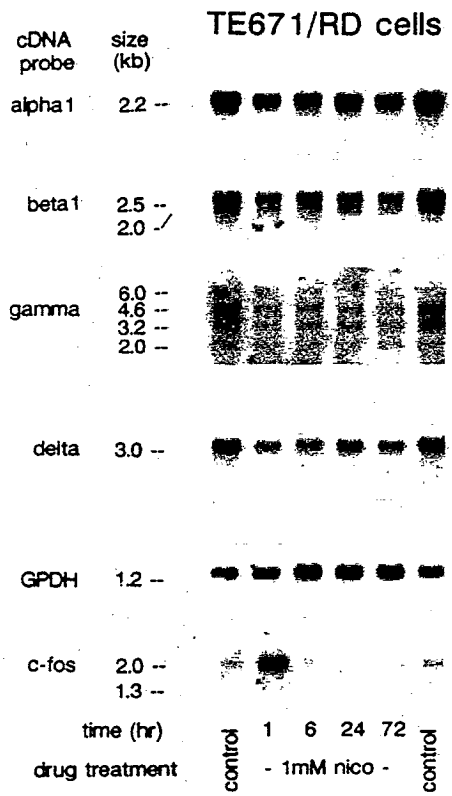


FIGURE 2. Temporal pattern of the effects of nicotine treatment on muscle-type nAChR subunit mRNA levels in TE671/RD cells. Northern blot analyses were conducted using established techniques and cDNA probes previously described[22,53] for TE671/RD cells that had been treated for the indicated periods with 1 mM nicotine (nico) or with no added drug (control). Approximate sizes (kb) of nAChR subunit transcripts corresponding to α1, β1, γ, and δ genes are shown as is mRNA for glyceraldehyde-3-phosphate dehydrogenase (GPDH), which was used as a control for mRNA loading. Quantitative densitometric analysis of this and other experiments indicates that there are no significant increases in nAChR subunit mRNA levels in nicotine-treated cells relative to drug-free controls when data are normalized to levels of GPDH or total (measured by densitometric analysis of photographic negatives of ethidium-stained mRNA in the smear between 18S and 28S ribosomal RNAs) mRNA. Note that nicotine treatment also transiently induces expression of c-fos mRNA.

transient effect of nicotine treatment on nAChR mRNA levels and probably on nAChR gene transcription. Our findings also reveal for the first time, in the absence of possible pharmacokinetic effects, that whereas chronic nicotine treatment can induce comparable increases in muscle-type nAChR, ganglia-type nAChR, or CNS α4/β2-nAChR, the dose of nicotine required to affect those changes differs from one nAChR subtype to another. We hypothesize that chronic nicotine exposure first induces a loss of nAChR function that somehow is sensed by the cell and/or triggers an increase in nAChR expression measurable by radioligand binding, but that this increase does not involve activation of nAChR subunit gene transcription. Continued studies are necessary to reveal the possible posttranslational mechanisms (enhanced assembly of nAChR subunits into detectable nAChR or enhanced recruitment of nAChR to the cell surface from a microsomal pool) involved in these effects. However, the current work illustrates how mechanisms of nAChR regulation can sometimes be blind to nAChR diversity. Moreover, these new findings indicate that nAChR diversity can contribute to differences in ligand sensitivity, not only in regard to concentrations of drug needed to acutely trigger nAChR function, but also to those needed to chronically alter nAChR expression and/or function. That the predominant brain nAChR subtype (but not muscle- or ganglia-type nAChR) is

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affected by concentrations of nicotine known to be present in the blood of smokers suggests that the current findings are relevant to the process of nicotine addiction as an event of the CNS and helps to explain why chronic nicotine exposure at levels seen in smokers is comparatively ineffective in chronically altering neuromuscular or ganglionic function.

## EFFECTS OF NERVE GROWTH FACTOR

Previous reports in the literature indicate that treatment of cells of the PC12 rat pheochromocytoma with nerve growth factor (NGF) causes an increase in nAChR function[47–49] and numbers,[50,51] and a recent study indicated that those effects are accompanied by an increase in levels of nAChR α3, α5, α7, β2, and β4 subunit mRNA levels.[52] Cells of the SH-SY5Y human neuroblastoma express the same range of nAChR subunits and subtypes as are expressed in PC12 cells or non-neoplastic autonomic neurons of neural crest origin.[22] As shown in FIGURE 3, new results indicate that levels of nAChR α3, α5, and β4 subunit mRNA increase in SH-SY5Y cells treated for as little as six hours or for as long as five days with human

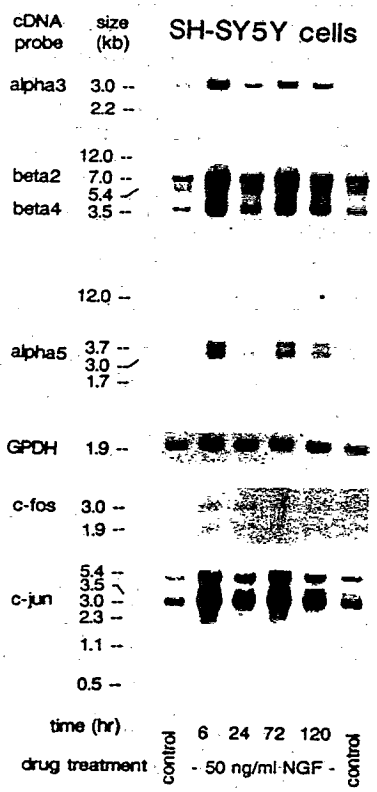


FIGURE 3. Temporal pattern of the effects of nerve growth factor (NGF) treatment on nAChR subunit mRNA levels in SH-SY5Y cells. Northern blot analyses were conducted using established techniques and cDNA probes previously described[22,53] for SH-SY5Y cells that had been treated for the indicated periods with 50 ng/mL NGF or with no added growth factor (control). Approximate sizes (kb) of nAChR subunit transcripts corresponding to α3, β2, β4, and α5 genes are shown as is mRNA for glyceraldehyde-3-phosphate dehydrogenase, which was used as a control for mRNA loading. Note also that there is sustained elevation of both c-fos and c-jun mRNAs on NGF treatment.

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recombinant NGF (50 ng/mL). Quantitative densitometric analysis indicates that about a threefold increase in mRNA levels occurs for the indicated subunits, but not for β2 subunit mRNA, levels of which matched those of the loading control (glyceraldehyde-3-phosphate dehydrogenase; GPDH). These results indicate that NGF treatment coordinately increases the expression in human SH-SY5Y cells of the three neuronal nAChR subunit genes that are found as a gene cluster in chickens and rats, as occurs upon treatment with phorbol ester (see below). This observation supports the hypothesis that the gene cluster acts as a functional unit.[20–21] The finding that human β2 gene expression in SH-SY5Y cells is not altered by NGF treatments that affect rat β2 gene expression in PC12 cells[52] (see also ref. 31) indicates that this gene can be uncoupled from the others. It also suggests that differences must exist in sequences of human and rat β2 genes in a region(s) that confers responsiveness to nuclear transacting factors sensitive to actions of NGF and perhaps other agents. A prediction of the current results is that ganglia-type nAChR numbers and function will be increased in SH-SY5Y cells subjected to NGF treatment. Given that NGF effects on nAChR subunit mRNA levels occur in wild-type and protein kinase A-deficient PC12 cells[52] and that both NGF and phorbol ester treatments have the same effects on nAChR subunit mRNA levels in SH-SY5Y cells (see below), another prediction is that the protein kinase C signaling pathway may mediate some of the effects of NGF or that the NGF and C-kinase signaling pathways converge at some point proximal to the activation of nAChR subunit gene expression. The current results illustrate how nAChR subunit diversity across species may influence responses of nAChR to regulatory influences, indicate that nAChR levels can be altered by pretranslational mechanisms, and demonstrate that growth factors can profoundly influence nAChR expression.

## POSSIBLE ROLE FOR THE CYTOSKELETON IN nAChR REGULATION

One question posed above was, How might a cell sense a change in its environment, and how could it transmit signals to alter expression or function of nAChR? The classical picture of the role of the cytoskeleton in receptor biology is one of a relatively passive, structural element that maintains cell shape and possibly participates in transport and local organization of receptors, perhaps in coordination with outgrowth of dendritic or axonal processes. However, some of our recent work,[53] particularly from the perspective of other reports in the literature, suggests that the cytoskeleton may play a more dynamic and functional role in the regulation of nAChR expression and function. Treatment of TE671/RD cells with any one of a variety of cytochalasins (done to exclude possible effects of a narrower subset of cytochalasins on cell functions or processes other than stability of actin microfilaments) produces changes in cell size and shape consistent with disruption of actin microfilament networks, that is, a rapid loss (within an hour) of submembrane, phalloidin staining of F-actin fibers coordinate with condensation of the cell into a rounded shape, followed by enlargement of the cell soma and bipolar process outgrowth apparently driven by extensive polymerization and parallel organization of tubulin filaments, clearly evident within two days of the start of cytochalasin treatment (R.J. Lukas, unpublished results). Cytochalasin treatment also produces a steady increase over four days of drug treatment in muscle-type nAChR numbers (i.e., I-Bgt binding sites) in total membrane fractions, but not on the cell surface.[53] nAChR function also declines (detectably so within hours), but 3–5-fold increases occur in levels of nAChR α1, β1, γ, and δ subunit mRNA levels at two days of cytochalasin treatment. All of these effects appear to be specific, in that levels of

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other mRNAs and numbers of other receptors (e.g., m3-type muscarinic acetylcholine receptors) are not affected. These results suggest that actin microfilament integrity is necessary for maintenance of nAChR function. They also suggest that actin microfilament integrity may play a role in tonic inhibition of nAChR subunit gene expression, perhaps providing a mechanism for transmitting signals (possibly to the nucleus) concerning the status of the cell membrane and its receptors.[53] Given that process outgrowth/retraction in neurons can be influenced by neurotransmitters including those that act at nAChR[54,55] and that those processes must involve reorganization of the cytoskeleton, it is clear that cytoskeletal structure and nAChR activity and expression can influence and be influenced by each other.[53] These studies suggest, for example, that synaptic remodeling may be a constant in the nervous system, influenced by interacting receptor activity and perhaps mediated by the cytoskeleton as well as by more mobile and compact signaling molecules. Another area ripe for investigation is the role that specific actin- or tubulin-associated proteins play as chaperons targeting nAChR via their unique cytoplasmic domains to different cellular destinations and as mediators of receptor-cytoskeletal signaling.

## ACTIONS ON nAChR EXPRESSION OF AGENTS TARGETING THE NUCLEUS

As a model for studies of extra- or intracellular signals that might be involved in mediating the presumably neuronal influences on nAChR gene expression in developing muscle, we initially investigated the effects of dibutyryl cyclic AMP (dbcAMP) on muscle-type nAChR and nAChR subunit gene expression in $BC_3H$-1 cells.[56] We found that dbcAMP treatment induced a dramatic loss in numbers of muscle-type nAChR (i.e., I-Bgt binding sites) and nAChR function. Northern blot analysis indicated that substantial (60–80%) declines occurred in levels of nAChR $\alpha 1$, $\beta 1$, and $\delta$ subunit mRNA, just as occurs in normal muscle about the time of neuronal innervation, but also that there was a virtually complete loss (quantifiable as a 250-fold decline, also as occurs in normal muscle about the time of neuronal innervation) in $\gamma$ subunit mRNA levels. The loss in $\gamma$ subunit gene expression clearly accounted for the loss of nAChR numbers and function, suggesting that gamma-less nAChR expression was very inefficient, at best, and that dbcAMP treatment may access a signaling pathway that is involved in the innervation-induced changes in nAChR subunit and phenotype expression *in vivo*. Studies of the time course for these effects and pharmacological dissection indicated, however, that the effects of dbcAMP in $BC_3H$-1 cells on nAChR subunit gene expression were attributable to the actions of butyrate, which must be generated in $BC_3H$-1 cells treated with dbcAMP by hydrolysis of dbcAMP to butyrate and monobutyryl cyclic AMP. Butyrate is known to specifically alter the expression of several genes, but the mechanism for those effects is presently being debated.[57,58] Among the possibilities are the effects of butyrate on histone acetylation, thereby affecting nucleosome structure, or on transacting or transcriptional factors; putative butyrate response elements have been identified in promoter/enhancer regions of viral genes, for example. The $BC_3H$-1 butyrate model provides an opportunity to investigate mechanisms of action of butyrate as well as possible nuclear mechanisms involved in the nAChR $\gamma/\epsilon$ subunit switch. These studies illustrate how nAChR subunit gene expression can be regulated differentially and how expression of nAChR can be influenced by agents that perhaps are specifically targeted to the nucleus.

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## SECOND MESSENGER REGULATION OF nAChR EXPRESSION

An obvious potential mechanism for the control of nAChR expression and function, particularly in the light of reports in the literature about the roles of nAChR phosphorylation in functional desensitization and in posttranslational processing of nAChR, is via second messengers that alter protein kinase activity.[2,59] Some of our previous studies have shown, for example, that muscle-type nAChR expression in human TE671/RD cells is subject to second messenger-sensitive

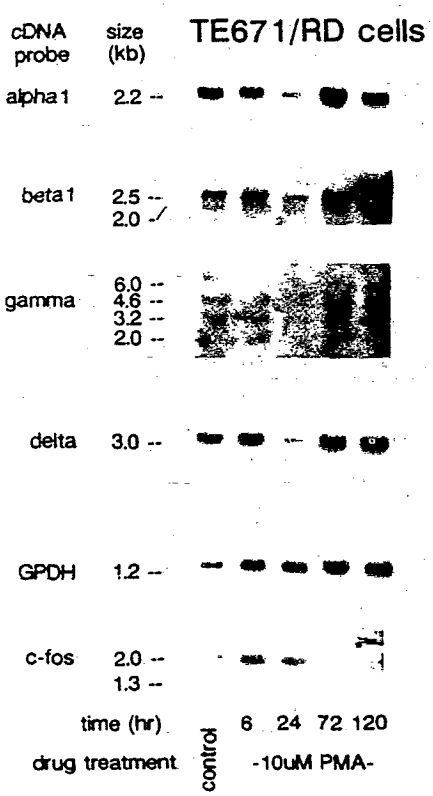


**FIGURE 4.** Temporal pattern of the effects of phorbol-12-myristate-13-acetate (PMA) treatment on muscle-type nAChR subunit mRNA levels in TE671/RD cells. Northern blot analyses were conducted using established techniques and cDNA probes previously described[22,53] for TE671/RD cells that had been treated for the indicated periods with 10 μM PMA or with no added drug (control). Approximate sizes (kb) of nAChR subunit transcripts corresponding to α1, β1, γ, and δ genes are shown, as is mRNA for glyceraldehyde-3-phosphate dehydrogenase, which was used as a control for mRNA loading. Note that PMA treatment also transiently elevates c-fos mRNA levels.

regulation.[60–61] Here, we describe and present Northern analysis data indicating that some, but not all, of these effects have a potential transcriptional basis. Treatment of TE671/RD cells with phorbol-12-myristate-13-acetate (PMA) induces a temporally biphasic effect on nAChR expression (i.e., I-Bgt binding sites in total membrane preparations) characterized by a transient loss of nAChR followed by an increase to 200% of control levels after 4–5 days of drug treatment. By contrast, treatment with dbcAMP (which does not seem to lead to production of butyrate in these cells),

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induces little-to-no change in nAChR numbers. Whereas Northern analysis reveals no change in muscle-type nAChR subunit mRNA levels in dbcAMP-treated TE671/RD cells, in cells treated with PMA, levels of mRNA corresponding to α1, β1, γ, and δ subunits follow the same temporal pattern of an early decline followed by an up-regulation as is seen for I-Bgt binding sites (FIG. 4). Hence, in this case, it is likely that a protein kinase C-sensitive pathway produces changes in nAChR subunit gene transcription to influence nAChR numbers. Interestingly, treatment of SH-SY5Y cells with PMA also produces a longer-term increase (of about 4–5-fold but without a transient decline as seen in TE671/RD cells) in transcript levels corresponding to nAChR α3, α5, and β4 subunits (but not β2 subunits; data not shown), suggesting that a variety of nAChR genes may be sensitive to protein kinase C-mediated signals. Collectively, these studies are consistent with posttranslational effects of protein kinase A-mediated signaling on nAChR and with both transcriptional and posttranslational effects (not discussed here) of protein kinase C-mediated signaling.

## SUMMARY

In our studies we explored the functional relevance of nAChR diversity, in part from the perspective of nAChR as ideal targets for regulatory influences, including those mediated via actions of ligands at other "interacting" receptors. We explored possible mechanisms for nAChR regulation and roles played by nAChR subtype and subunit diversity in those processes. We showed that regulatory factors can influence nAChR numbers at transcriptional and posttranscriptional levels and can affect nAChR function and subcellular distribution. We also demonstrated that nAChR expression can be influenced (1) by nicotinic ligands, (2) by second messengers, (3) by growth factors, (4) by agents targeting the nucleus, and (5) by agents targeting the cytoskeleton. We found common effects of some regulatory influences on more than one nAChR subtype, and we found instances where regulatory influences differ for different cell and nAChR types. Even from the very limited number of these initial studies, it is evident that nAChR subunit and subtype diversity, which alone can provide diversity in nAChR functions, localization, and ligand sensitivity, dovetails with diversity in cellular signaling mechanisms that can affect nAChR expression to amplify the potential functional plasticity of cholinoceptive cells. As examples, we discussed potential roles for nAChR diversity and regulatory plasticity in synapse remodeling and in changes in neuronal circuit conditions. These examples illustrate how nAChR diversity could play important roles in the regulation of nervous system function.

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